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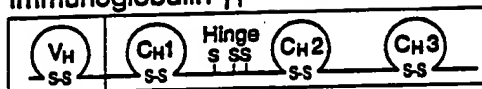
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(54) Title: ADHESION VARIANTS

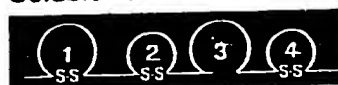
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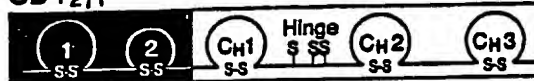
Immunoglobulin  $\gamma_1$



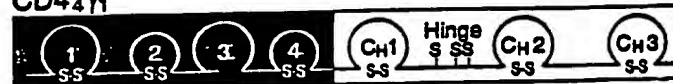
Soluble rCD4



CD4  $\gamma_1$



CD4  $\gamma_1$



(57) Abstract

Novel derivatives of cell surface proteins which are homologous to the immunoglobulin superfamily (adhesions) are provided. Amino acid sequence variations are introduced into adhesion, the most noteworthy of which are those in which the transmembrane and, preferably, cytoplasmic domains are rendered functionally inactive, and in which adhesion extracellular domains replace an immunoglobulin variable region. These variants are useful in therapy or diagnostics, in particular, CD4 variants are therapeutically useful in the treatment of HIV infections.

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## ADHESION VARIANTS

Background of the Invention

5 This application relates to compositions for antiviral or immunomodulatory therapy. In particular, it relates to compositions useful in the treatment of Human Immunodeficiency Virus (HIV) infections.

10 The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (H. Lane *et al.*, *Ann. Rev. Immunol.* 3:477 [1985]). CD4 is a non-polymorphic glycoprotein with homology to the immunoglobulin gene superfamily (P. Maddon *et al.*, *Cell* 42:93  
15 [1985]). Together with the CD8 surface antigen, CD4 defines two distinct subsets of mature peripheral T cells (E. Reinherz *et al.*, *Cell* 19:821 [1980]), which are distinguished by their ability to interact with nominal antigen targets in the context of class I and class II major histocompatibility complex (MHC) antigens, respectively (S. Swain, *Proc. Natl. Acad. Sci.* 78:7101 [1981]; E. Engleman *et al.*, *J. Immunol.* 127:2124 [1981]; H. Spitz *et al.*, *J. Immunol.* 129:1563 [1982]; W. Biddison *et al.*, *J. Exp. Med.* 156:1065 [1982]; and D. Wilde *et al.*, *J. Immunol.* 131:2178 [1983]). For the  
20 most part, CD4 T cells display the helper/inducer T cell phenotype (E. Reinherz, *supra*), although CD4 T cells characterized as cytotoxic/suppressor T cells have also been identified (Y. Thomas *et al.*, *J. Exp. Med.* 154:459 [1981]; S. Meuer *et al.*, *Proc. Natl. Acad. Sci. USA* 79:4395 [1982]; and A. Krensky *et al.*, *Proc. Natl. Acad. Sci. USA* 79:2365 [1982]). The loss of CD4 helper/inducer T  
25 cell function probably underlies the profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of the acquired immunodeficiency syndrome (AIDS) (H. Lane *supra*).

30

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Studies of HIV-I infection of fractionated CD4 and CD8 T cells from normal donors and AIDS patients have revealed that depletion of CD4 T cells results from the ability of HIV-I to selectively infect, replicate in, and ultimately destroy this T lymphocyte subset (D. Klatzmann *et al.*, Science 225:59 [1984]). The possibility that CD4 itself is an essential component of the cellular receptor for HIV-I was first indicated by the observation that monoclonal antibodies directed against CD4 block HIV-I infection and syncytia induction (A. Dalgleish *et al.*, Nature [London] 312:767 [1984]; J. McDougal *et al.*, J. Immunol. 135:3151 [1985]). This hypothesis has been confirmed by the demonstration that a molecular complex forms between CD4 and gp120, the major envelope glycoprotein of HIV-I (J. McDougal *et al.*, Science 231:382 [1986]; and the finding that HIV-I tropism can be conferred upon ordinarily non-permissive human cells following the stable expression of a CD4 cDNA (P. Maddon *et al.*, Cell 47:333 [1986]). Furthermore, the neurotropic properties of HIV-I, reflected by a high incidence of central nervous system dysfunction in HIV-I infected individuals (W. Snider *et al.*, Ann. Neurol. 14:403 [1983]), and the ability to detect HIV-I in the brain tissue and cerebrospinal fluid of AIDS patients (G. Shaw *et al.*, Science 227:177 [1985]; L. Epstein, AIDS Res. 1:447 [1985]; S. Koenig, Science 233:1089 [1986]; D. Ho *et al.*, N. Engl. J. Med. 313:1498 [1985]; J. Levy *et al.*, Lancet II:586 [1985]), appears to have its explanation in the expression of CD4 in cells of neuronal, glial and monocyte/macrophage origin (P. Maddon, Cell 47:444 [1986]; I. Funke *et al.*, J. Exp. Med. 165:1230 [1986]; B. Tourville *et al.*, Science 234:610 [1986]).

In addition to determining the susceptibility to HIV-I infection, the manifestation of cytopathic effects in the infected host cell appears to involve CD4. Antibody to CD4 was found to inhibit the fusion of uninfected CD4 T cells with HIV-I infected cells *in vitro*; moreover, the giant multinucleated cells produced by this event die shortly after being formed resulting in the

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depletion of the population of CD4 cells (J. Lifson *et al.*, Science 232:1123 [1986]). Formation of syncytia also requires gp120 expression, and can be elicited by coculturing CD4-positive cell lines with cell lines expressing the HIV-I *env* gene in the absence of other viral structural or regulatory proteins (J. Sodroski *et al.*, Nature 322:470 [1986]; J. Lifson *et al.*, Nature 323:725 [1986]). Thus, in mediating both the initial infection by HIV-I as well as eventual cell death, the interaction between gp120 and CD4 constitutes one of several critical entry points in the viral life cycle amenable to therapeutic intervention (H. Mitsuya *et al.*, Nature 325:773 [1987]).

The known sequence of the CD4 precursor predicts a hydrophobic signal peptide, an extracellular region of approximately 370 amino acids, a highly hydrophobic stretch with significant identity to the membrane-spanning domain of the class II MHC beta chain, and a highly charged intracellular sequence of 40 residues (P. Madden, Cell 42:93 [1985]). The extracellular domain of CD4 consists of four contiguous regions each having amino acid and structural similarity to the variable and joining (V-J) domains of immunoglobulin light chains as well as related regions in other members of the immunoglobulin gene superfamily (a subclass of which are defined herein by the coined term "adhesons". These structurally similar regions of CD4 are termed the V<sub>1</sub>, V<sub>2</sub>, V<sub>3</sub> and V<sub>4</sub> domains (denominated 1-4 in Fig. 3).

A successful strategy in the development of drugs for the treatment of many receptor mediated abnormalities has been the identification of antagonists which block binding of the natural ligand. Since the CD4 adhesion ordinarily binds to the recognition sites of the HIV envelope it would appear to be a candidate for therapeutically sequestering these HIV sites, thereby blocking viral infectivity. However, full length CD4 and other adhesons are cell membrane proteins which are anchored in the lipid bilayer of cells. The presence of membrane components will be undesirable

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from the standpoint of manufacturing and purification. In addition, since adhesions are normally present only on cell surfaces, it would be desirable to produce adhesions in a form which is more stable in the circulation. Additionally, even truncated, soluble CD4 adhesion (generally referred to as CD4T) may not be optimally effective as a therapeutic since it possesses a relatively short biological half-life, binds to HIV no better than cell surface CD4, may not cross the placental or other biological barriers and since it merely sequesters the HIV recognition sites without in itself bearing an infected-cell killing or virus killing functionality.

Accordingly, it is an object of this invention to produce soluble, secreted adhesions. It is another object to produce CD4 derivatives useful in the treatment of AIDS and related conditions, in a manner essentially unaffected by the extreme degree of genetic variation observed among various HIV-I isolates and their respective env polypeptides (J.Coffin, Cell 46:1 [1986]). Still another object is to prepare adhesions fused to other polypeptides in order to provide molecules with novel functionalities such as those described above for therapeutic use, or diagnostic reagents for the *in vitro* assay of adhesions or their ligands. In particular, it is an objective to prepare molecules for directing toxins or effector molecules (for example the Fc domain of immunoglobulin) to cells bearing receptors for the adhesions, e.g. HIV gp120 in the case of CD4, and for use in facilitating purification of the adhesions. It is a further object to provide stable, highly purified adhesion preparations.

30

#### Summary

The objects of this invention are accomplished by providing nucleic acid encoding an amino acid sequence variant of an adhesion, in particular a variant in which the trans-membrane domain is modified so that it is no longer capable of becoming lodged in the

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cell membrane. In the case of CD4 such variants are termed soluble CD4.

5 Variant adhesions are produced by a method comprising (a) transforming a host cell with nucleic acid encoding an amino acid sequence variant of an adhesion, (b) culturing the host cell and (c) recovering the variant adhesion from the host cell culture media or from lysates of the host cell.

10 In specific embodiments, the objects of this invention are accomplished by providing an adhesion variant selected from the group consisting of (a) an adhesion amino acid sequence variant having an inactivated transmembrane domain and (b) a polypeptide comprising an adhesion extracellular domain fused to the sequence of  
15 a polypeptide which is different from the adhesion, this latter, for example, selected from a cytotoxin, an immunogen or a protein with a long plasma half life such as an immunoglobulin constant domain.

20 In a preferred embodiment a polypeptide comprising a gp120 binding domain of the CD4 adhesion is fused at its C-terminus to an immunoglobulin constant domain, or is linked to a cytotoxic polypeptide such as ricin.

25 The CD4 adhesion variants provided herein are purified and formulated in pharmacologically acceptable vehicles for administration to patients in need of antiviral, neuromodulatory or immunomodulatory therapy, in particular patients infected with HIV, and for use in the modulation of cell adhesion.

30 Brief Description of the Drawings

Figs. 1a-1c depict the amino acid and nucleotide sequence of a secreted form of the CD4 adhesion. The signal processing site is designated with an arrow.



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Figs. 2a-2c depict the amino acid and nucleotide sequence of a fusion of the herpes gD leader and N-terminal 27 residues to the putative mature N-terminus of CD4T.

5            Fig. 3 depicts the structural elements of the native and soluble CD4 adhesion, the native human IgG<sub>1</sub> ( $\gamma_1$ ) heavy chain and two exemplary heavy chain-CD4 chimeras.

10           Figs. 4a-4b are a map of the linkered human IgG<sub>1</sub> ( $\gamma_1$ ) chain fragment employed in the preparation of CD4 fusions. Insert sites are designated  $\gamma_1$  and Fc.

15           Fig. 5 is a map of the human  $\kappa$  light chain fragment useful for CD4 fusions at the arrow flanked by V $\kappa$ J $\kappa$  (light variable and joining) and C $\kappa$  (light constant).

#### Detailed Description

Adhesions are cell surface polypeptides having an extracellular domain which is homologous to a member of the immunoglobulin gene superfamily, excluding, however, highly polymorphic members of this superfamily selected from the group of class I and class II major histocompatibility antigens, immunoglobulins and T-cell receptor  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  chains. Examples of adhesions include CD1, CD2, CD4, CD8, CD28, the  $\gamma$ ,  $\delta$  and  $\epsilon$  chains of CD3, OX-2, Thy-1, the intercellular or neural cell adhesion molecules (I-CAM or N-CAM), lymphocyte function associated antigen-3 (LFA-3), neurocytoplasmic protein (NCP-3), poly-Ig receptor, myelin-associated glycoprotein (MAG), high affinity IgE receptor, the major glycoprotein of peripheral myelin (Po), platelet derived growth factor receptor, colony stimulating factor-1 receptor, macrophage Fc receptor, Fc gamma receptors and carcinoembryonic antigen. Homologous as defined herein means having the sequence of a member of the immunoglobulin gene superfamily or having a sequence therewithin which has substantially the same as (or a greater degree of) amino acid

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sequence homology to a known member of the superfamily as the specific examples given above have to the sequence of an immunoglobulin variable or constant domain. Preferred adhesons are CD4, CD8 and high affinity IgE Fc receptor.

5

This invention is particularly concerned with amino acid sequence variants of adhesons. Amino acid sequence variants of adhesons are prepared with various objectives in mind, including increasing the affinity of the adheson for its binding partner, facilitating the stability, purification and preparation of the adheson, increasing its plasma half life, improving therapeutic efficacy as described above in the background, introducing additional functionalities and lessening the severity or occurrence of side effects during therapeutic use of the adheson. Amino acid sequence variants of adhesons fall into one or a combination of the following classes: insertional, substitutional or deletional variants.

Insertional amino acid sequence variants are those in which one or more amino acid residues extraneous to the adheson are introduced into a predetermined site in the adheson including the C or N termini. Such variants are referred to as fusions of the adheson and a different polypeptide. Such other polypeptides contain sequences other than those which are normally found in the adheson at the inserted position. Several groups of fusions are contemplated herein. Immunologically active adheson fusions comprise an adheson and a polypeptide containing a non-adheson epitope. The non-adheson epitope is any immunologically competent polypeptide, i.e., any polypeptide which is capable of eliciting an immune response in the animal to which the fusion is to be administered or which is capable of being bound by an antibody raised against the non-adheson polypeptide. Typical non-adheson epitopes will be those which are borne by allergens, autoimmune epitopes, or other potent immunogens or antigens recognized by pre-existing antibodies in the fusion recipient, including bacterial

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polypeptides such as trpLE, beta-galactosidase, viral polypeptides such as herpes gD protein, and the like. Immunogenic fusions are produced by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding an immunogenic polypeptide. It is preferable that the immunogenic fusion be one in which the immunogenic sequence is joined to or inserted into the adhesion antigen or fragment thereof by a peptide bond(s). These products therefore consist of a linear polypeptide chain containing adhesion epitopes and at least one epitope foreign to the adhesion. It will be understood that it is within the scope of this invention to introduce the epitopes anywhere within the adhesion molecule or fragment thereof. Such fusions are conveniently made in recombinant host cells or by the use of bifunctional cross-linking agents. The use of a cross-linking agent to fuse the adhesion to the immunogenic polypeptide is not as desirable as a linear fusion because the cross-linked products are not as easily synthesized in structurally homogeneous form.

These immunogenic insertions are particularly useful when formulated into a pharmacologically acceptable carrier and administered to a subject in order to raise antibodies against the adhesion, which antibodies in turn are useful in diagnostics or in purification of adhesion by immunoaffinity techniques known *per se*. Alternatively, in the purification of adhesions, binding partners for the fused non-adhesion polypeptide, e.g. antibodies, receptors or ligands, are used to adsorb the fusion from impure admixtures, after which the fusion is eluted and, if desired, the adhesion is recovered from the fusion, e.g. by enzymatic cleavage.

Other fusions, which may or may not also be immunologically active, include fusions of the adhesion sequence with a signal sequence heterologous to the adhesion, fusions of transmembrane-modified CD4 adhesions, for example, to polypeptides having enhanced plasma half life (ordinarily >about 20 hours) such as immunoglobulin chains or fragments thereof, and fusions with

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cytotoxic functionalities. Signal sequence fusions are employed in order to more expeditiously direct the secretion of the adheson. The heterologous signal replaces the native adheson signal, and when the resulting fusion is recognized, i.e. processed and cleaved by the host cell, the adheson is secreted. Signals are selected based on the intended host cell, and may include bacterial yeast, mammalian and viral sequences. The herpes gD glycoprotein signal is suitable for use in mammalian expression systems.

Plasma proteins which have enhanced plasma half-life longer than that of transmembrane modified CD4 include serum albumin, immunoglobulins, apolipoproteins, and transferrin. Preferably, the adheson-plasma protein fusion is not significantly immunogenic in the animal in which it is used and the plasma protein does not cause undesirable side effects in patients by virtue of its normal biological activity.

In a specific embodiment the adheson immunoglobulin-like domain which may be homologous either to the constant or to the variable region domains is conjugated with an immunoglobulin constant region sequence. The resulting products are referred to herein as immuno adhesons. Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner et al., Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler et al., P.N.A.S. USA 77:2197 (1980); Raso et al., Cancer Res. 41:2073 (1981); Morrison et al., Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison et al., P.N.A.S. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

Ordinarily, the domains of adhesons that are homologous to immunoglobulins and extracellular in their native environment are

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fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s) thereof, retaining at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. This  
5 ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Immunoglobulins and other polypeptides having enhanced plasma half life are fused to the extracellular or ligand binding domains of other adhesons in the same fashion.

10

The boundary domains for the CD4 V-like regions (V1-V4) are, respectively, about 100-109, about 175-184, about 289-298, and about 360-369 (based on the precursor CD4 amino acid sequence in which the initiating met is -25; Fig. 1a). CD4 sequences  
15 containing any of the CD4 V domains are fused to the immunoglobulin sequence. It is preferable that the V1V2 or V1V2V3V4 be fused at their C-termini to the immunoglobulin constant region. The precise site at which the fusion is made is not critical; the boundary domains noted herein are for guidance only and other sites  
20 neighboring or within the V regions may be selected in order to optimize the secretion or binding characteristics of the CD4. The optimal site will be determined by routine experimentation. In general, it has been found that the fusions are expressed intracellularly, but a great deal of variation is encountered in  
25 the degree of secretion of the fusions from recombinant hosts. For instance, the following table demonstrates the various immunoglobulin fusions that have been obtained by the method of this invention. In all examples of CD4 immunoadhesons, the CD4 signal was used to direct secretion from 293 cells. Lower case m represents murine origin, while the lower case h designates human origin. V and C are abbreviations for immunoglobulin variable and constant domains respectively. The numerical subscripts indicate  
30 the number of parenthetical units found in the designated multimer. It will be understood that the chains of the multimers are believed  
35 to be disulfide bonded in the same fashion as native

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immunoglobulins. The CD4 immunoadhesons typically contained either the first N-terminal 366 residues of CD4 (CD4<sub>4</sub>) or the first 180 N-terminal residues of CD4 (CD4<sub>2</sub>) linked at their C-terminus to the  $\kappa$  (light) chain or IgG1 heavy chain constant region ( $\gamma$ 1).

5

Table I

	<u>Transfected Gene</u>	<u>Secreted Product</u>
	mV $\kappa$ C $\kappa$	mV $\kappa$ C $\kappa$ and/or (mV $\kappa$ C $\kappa$ ) <sub>2</sub>
10	mV $\gamma$ 1C $\gamma$ 1	ND
	mV $\kappa$ C $\kappa$ + mV $\gamma$ 1C $\gamma$ 1	(mV $\kappa$ C $\kappa$ ) <sub>2</sub> (mV $\gamma$ 1C $\gamma$ 1) <sub>2</sub> + mV $\kappa$ C $\kappa$ and/or (mV $\kappa$ C $\kappa$ ) <sub>2</sub>
15	hCD4-mC $\kappa$	hCD4-mC $\kappa$ and/or (hCD4-mC $\kappa$ ) <sub>2</sub>
	hCD4-mC $\gamma$ 1	ND
20	hCD4-mC $\kappa$ + hCD4-mC $\gamma$ 1	(hCD4-mC $\kappa$ ) <sub>2</sub> (hCD4-mC $\gamma$ 1) <sub>2</sub> + hCD4-mC $\kappa$ and/or (hCD4-mC $\kappa$ ) <sub>2</sub>
	hCD4-hC $\kappa$	hCD4-hC $\kappa$ and/or (hCD4-hC $\kappa$ ) <sub>2</sub>
	hCD4-hC $\gamma$ 1	(hCD4-hC $\gamma$ 1) <sub>2</sub>
25	hCD4-hC $\kappa$ + hCD4-hC $\gamma$ 1	(hCD4-hC $\kappa$ ) <sub>2</sub> (hCD4-hC $\gamma$ 1) <sub>2</sub> + hCD4-hC $\kappa$ and/or (hCD4-hC $\kappa$ ) <sub>2</sub>
30	mV $\kappa$ C $\kappa$ + hCD4-hC $\gamma$ 1	(mV $\kappa$ C $\kappa$ ) <sub>2</sub> (hCD4-hC $\gamma$ 1) <sub>2</sub> + mV $\kappa$ C $\kappa$ and/or (mV $\kappa$ C $\kappa$ ) <sub>2</sub>

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\*ND = Not detected

It is interesting to observe from this table that the CD4-human heavy chain immunoadereson was secreted as a dimer whereas the analogous murine construction was not detected (this not excluding

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the intracellular accumulation of the protein, however). The ability of the hCD4-hC $\gamma$ 1 transformants to produce heavy chain dimer was unexpected since previous work had suggested that immunoglobulin heavy chains are not secreted unless the hosts are cotransformed with nucleic acid encoding both heavy and light chain (Valle et al., Nature 241:338 [1981]). According to this invention, CD4-IgG immunoadheson chimeras are readily secreted wherein the CD4 epitope is present in heavy chain dimers, light chain monomers or dimers, and heavy and light chain heterotetramers wherein the CD4 epitope is present fused to one or more light or heavy chains, including heterotetramers wherein up to and including all four variable region analogues are derived from CD4. Where light-heavy chain non-CD4 variable domain is present, a heterofunctional antibody thus is provided.

Various exemplary hetero-and chimeric immunoadheson antibodies produced in accordance with this invention are schematically diagrammed below. "A" means at least a portion of the extracellular domain of an adhesion containing its ligand binding site; V<sub>L</sub>, V<sub>H</sub>, C<sub>L</sub> and C<sub>H</sub> represent light or heavy chain variable or constant domains of an immunoglobulin; n is an integer; and Y designates a covalent cross-linking moiety.

- (a) A<sub>C<sub>L</sub></sub>;
- (b) A<sub>C<sub>L</sub></sub>-A<sub>C<sub>L</sub></sub>;
- (c) A<sub>C<sub>H</sub></sub>-[A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-V<sub>H</sub>C<sub>H</sub>, V<sub>L</sub>C<sub>L</sub>-A<sub>C<sub>H</sub></sub>, or V<sub>L</sub>C<sub>L</sub>-V<sub>H</sub>C<sub>H</sub>];
- (d) A<sub>C<sub>L</sub></sub>-A<sub>C<sub>H</sub></sub>-[A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-V<sub>H</sub>C<sub>H</sub>, V<sub>L</sub>C<sub>L</sub>-A<sub>C<sub>H</sub></sub>, or V<sub>L</sub>C<sub>L</sub>-V<sub>H</sub>C<sub>H</sub>];
- (e) A<sub>C<sub>L</sub></sub>-V<sub>H</sub>C<sub>H</sub>-[A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-V<sub>H</sub>C<sub>H</sub>, V<sub>L</sub>C<sub>L</sub>-A<sub>C<sub>H</sub></sub>, or V<sub>L</sub>C<sub>L</sub>-V<sub>H</sub>C<sub>H</sub>];
- (f) V<sub>L</sub>C<sub>L</sub>-A<sub>C<sub>H</sub></sub>-[A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-A<sub>C<sub>H</sub></sub>, A<sub>C<sub>L</sub></sub>-V<sub>H</sub>C<sub>H</sub>, V<sub>L</sub>C<sub>L</sub>-A<sub>C<sub>H</sub></sub>, or V<sub>L</sub>C<sub>L</sub>-V<sub>H</sub>C<sub>H</sub>];
- or
- (g) [A-Y]<sub>n</sub>-[V<sub>L</sub>C<sub>L</sub>-V<sub>H</sub>C<sub>H</sub>]<sub>2</sub>.

The structures shown in this table show only key features, e. g. they do not show joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. These are omitted in the interests of brevity. However, where such domains are

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required for binding activity they shall be construed as being present in the ordinary locations which they occupy in the adhesion, immunoadhesion or immunoglobulin molecules as the case may be. These examples are representative of divalent antibodies; more complex structures would result by employing immunoglobulin heavy chain sequences from other classes, e.g. IgM. The immunoglobulin  $V_LV_H$  antibody combining site also designated as the companion immunoglobulin, preferably is capable of binding to a predetermined antigen.

Suitable companion immunoglobulin combining sites and fusion partners are obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG-1.

A preferred embodiment is a fusion of an N-terminal portion of CD4, which contains the binding site for the gp120 envelope protein of HIV, to the C-terminal  $F_c$  portion of an antibody, containing the effector functions of immunoglobulin  $G_1$ . There are two preferred embodiments of this sort; in one, the entire heavy chain constant region is fused to a portion of CD4; in another, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG  $F_c$  chemically (residue 216, taking the first residue of heavy chain constant region to be 114 [Kabat et al., "Sequences of Proteins of Immunological Interest" 4th Ed., 1987], or analogous sites of other immunoglobulins) is fused to a portion of CD4. These embodiments are described in the examples.

More particularly, those variants in which one or more immunoglobulin-like domains of an adhesion are substituted for the variable region of an immunoglobulin chain are believed to exhibit improved *in vivo* plasma half life. These chimeras are constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; Munro, Nature 312: (13 December 1984); Neuberger et al.,



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Nature 312: (13 December 1984); Sharon *et al.*, Nature 309: (24 May 1984); Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Morrison *et al.* Science 229:1202-1207 (1985); and Boulianne *et al.*, Nature 312:643-646 (13 December 1984). The DNA encoding the adhesion immunoglobulin-like domain(s) is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the mature adhesion polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for the adhesion (where the native adhesion signal is employed). This DNA fragment then is readily inserted into DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, tailored by deletional mutagenesis. Preferably, this is a human immunoglobulin when the variant is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams *et al.*, Biochemistry 19:2711-2719 (1980); Gough *et al.*, Biochemistry 19:2702-2710 (1980); Dolby *et al.*, P.N.A.S. USA, 77:6027-6031 (1980); Rice *et al.*, P.N.A.S. USA 79:7862-7865 (1982); Falkner *et al.*, Nature 298:286-288 (1982); and Morrison *et al.*, Ann. Rev. Immunol. 2:239-256 (1984).

DNA encoding the immunoglobulin or immunoadhesion chimeric chain(s) is transfected into a host cell for expression. If the host cell is producing an immunoglobulin prior to transfection then one need only transfect with the adhesion fused to light or to heavy chain to produce a heteroantibody. The aforementioned immunoglobulins having one or more arms bearing the adhesion domain and one or more arms bearing companion variable regions result in dual specificity for adhesion ligand and for an antigen. These are produced by the above-described recombinant methods or by *in vitro* procedures. In the latter case, for example, F(ab')<sub>2</sub> fragments of the adhesion fusion and an immunoglobulin are prepared, the F(ab')<sub>2</sub> fragments converted to Fab' fragments by reduction under mild

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reducing conditions, and then reoxidized in each other's presence under acidic conditions in accord with methods known *per se*. See also U.S. patent 4,444,878.

5      Additionally, procedures are known for producing intact heteroantibodies from immunoglobulins having different specificities. These procedures are adopted for the *in vitro* production of heterochimeric antibodies by simply substituting the immunoadheson chains for one of the previously employed  
10      immunoglobulins.

         In an alternative method for producing a heterofunctional antibody, host cells producing an adhesion-immunoglobulin fusion, e.g. transfected myelomas, also are fused with B cells or  
15      hybridomas which secrete antibody having the desired companion specificity for an antigen. Heterobifunctional antibody is recovered from the culture medium of such hybridomas, and thus may be produced somewhat more conveniently than by conventional *in vitro* resorting methods (EP 68,763).

20      Another group of fusions are those in which an adhesion is conjugated with a toxic substance, e.g. a polypeptide such as ricin (including deglycosylated ricin A chain), diphtheria toxin A, or a non-peptidyl cytotoxin. Where the toxin is a polypeptide it is  
25      convenient to cross-link the polypeptide to the adhesion or its transmembrane-deleted variant by conventional *in vitro* protein cross-linking agents (for suitable methods for linking ricin A chain or deglycosylated A chain to CD4 see, for example, Duncan *et al.*, "Analy. Biochem." 132:68-73 [1983]; Thorpe *et al.*, "Cancer Res." 47:5924 [1987]; and Ghotie *et al.*, "Cancer Res." 48:2610  
30      [1988]) or by recombinant synthesis as a fusion (see for example, U.S. Patent 4,765,382). Alternatively, where companion antibodies are anti-ricin antibody immunoglobulin variable domains, such immunoglobulin heteroantibodies are employed to deliver ricin to

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HIV infected cells following the general procedure of Raso et al., Cancer Research, 41:2073 (1981).

Another class of adhesion variants are deletional variants. Deletions are characterized by the removal of one or more amino acid residues from a adhesion sequence. Typically, the transmembrane and cytoplasmic domains of adhesions are deleted. In the case of CD4, at least residues 368 to 395 (the transmembrane region), and ordinarily 396-433 as well (the cytoplasmic domain), will be deleted to obtain secreted forms of this adhesion. Parenthetically, the amino acid residues follow the numbers given for mature CD4 as noted, for example, in figures 1a - 1c. Thus, CD4T molecules generally will terminate in the vicinity of about residues 366-368, or at any other suitable site N-terminal thereto which preserves the gp120-binding capability of the CD4 variant.

Substitutional variants are those in which at least one residue in the adhesion sequence has been removed and a different residue inserted in its place. The native N-terminal residue for mature CD4 is now known to be lysine. Thus, the sequence shown in Fig. 1, with an N-terminal asparagine, is an amino acid sequence variant of native mature CD4. Table 2 below describes substitutions which in general will result in fine modulation of the characteristics of the CD antigen.

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TABLE 2

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
	Ala	ser
	Arg	lys
5	Asn	gln; his
	Asp	glu
	Cys	ser; ala
	Gln	asn
	Glu	asp
10	Gly	pro
	His	asn; gln
	Ile	leu; val
	Leu	ile; val
	Lys	arg; gln; glu
15	Met	leu; ile
	Phe	met; leu; tyr
	Ser	thr
	Thr	ser
	Trp	tyr
20	Tyr	trp; phe
	Val	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in adhesion properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteinyl or prolyl is substituted for (or by) any other residue; (c) a residue having

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an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanyl, is substituted for (or by) one not having a side chain, e.g., glycyl.

A preferred class of substitutional or deletional variants are those involving the transmembrane region of the adhesion. The transmembrane region of the adhesion is a highly hydrophobic or lipophilic domain that is the proper size to span the lipid bilayer of the cellular membrane. It is believed to anchor the adhesion in the cell membrane.

Deletion or substitution of the transmembrane domain will facilitate recovery and provide a soluble form of the adhesion by reducing its cellular or membrane lipid affinity and improving its water solubility. If the transmembrane and cytoplasmic domains are deleted one avoids the introduction of potentially immunogenic epitopes, either by exposure of otherwise intracellular polypeptides that might be recognized by the body as foreign or by insertion of heterologous polypeptides that are potentially immunogenic. A principal advantage of the transmembrane deleted adhesion is that it is secreted into the culture medium of recombinant hosts. This variant is water soluble and does not have an appreciable affinity for cell membrane lipids, thus considerably simplifying its recovery from recombinant cell culture.

It will be amply apparent from the foregoing discussion that substitutions, deletions, insertions or any combination thereof are introduced to arrive at a final construct. As a general proposition, all variants will not have a functional transmembrane domain and preferably will not have a functional cytoplasmic sequence. This is generally accomplished by deletion of the relevant domain, although adequate insertional or substitutional mutagens also can be effective for this purpose.

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For example, the transmembrane domain is substituted by any amino acid sequence, e.g. a random or homopolynucleic sequence of about 5 to 50 serine, threonine, lysine, arginine, glutamine, aspartic acid and like hydrophilic residues, which altogether exhibit a hydrophilic hydrophathy profile, so that it is secreted into the culture medium of recombinant hosts. This variant should also be considered to be an adhesion variant.

These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the adhesion, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant adhesions also are prepared by *in vitro* synthesis. Obviously, variations made in the DNA encoding the variant adhesions must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure deleterious to expression (EP 75,444A). The CD4 variants typically exhibit the same gp120 binding activity as does the naturally-occurring prototype, although variants also are selected in order to modify the characteristics of the CD4 adhesion as indicated above.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed adhesion variants screened for the optimal combination of desired activities. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis.

Adhesion variants that are not capable of binding HIV gp120 are useful nonetheless as immunogens for raising antibodies to the adhesion or as immunoassay kit components (labelled, as a competitive reagent for gp120 assay, or unlabelled as a standard

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for an adheson assay) so long as at least one adheson epitope remains active.

The DNA encoding adhesons is obtained by known procedures. See Williams, Immunol. Today 8:298-303 (1987) and citations therein. In general, prokaryotes are used for cloning of CD4 variant DNA sequences. For example, *E. coli* strain SR101 (for propagating ml3 phage, a  $\lambda$ -resistant strain of JM 101; Messing et al., Nucl. Acids. Res. 9(2):309-321 [1981]); and *E. coli* K12 strain 294 (ATCC No. 31446) are particularly useful. Other microbial strains which may be used include *E. coli* B, UM101 and *E. coli*  $\chi$ 1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

DNA encoding the variant adhesons are inserted for expression into vectors containing promoters and control sequences which are derived from species compatible with the intended host cell. The vector ordinarily, but need not, carry a replication site as well as one or more marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using a derivative of pBR322 which is a plasmid derived from an *E. coli* species (Bolivar, et al., Gene 2: 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA constructions.

Promoters suitable for use with prokaryotic hosts illustratively include the  $\beta$ -lactamase and lactose promoter systems (Chang et al., Nature, 275: 615 [1978]; and Goeddel et al., Nature 281: 544 [1979]), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res. 8: 4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the tac

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promoter (H. de Boer et al., Proc. Natl. Acad. Sci. USA 80: 21-25 [1983]). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to DNA encoding the adheson variant using linkers or adaptors to supply any required restriction sites (Siebenlist et al., Cell 20: 269 [1980]). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antigen.

In addition to prokaryotes, eukaryotic microbes such as yeast cultures also are useful as cloning or expression hosts. Saccharomyces cerevisiae, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb, et al., Nature 282: 39 [1979]; Kingsman et al, Gene 7: 141 [1979]; Tschemper et al., Gene 10: 157 [1980]) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85: 12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective means of selection by growth in the absence of tryptophan.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7: 149 [1968]; and Holland, Biochemistry 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.



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Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoters for controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. the beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers et al., Nature, 273: 113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway, P.J. et al., Gene 18: 355-360 (1982). Of course, promoters from the host cell or related species also are useful herein.

DNA transcription in higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300bp, that act to increase the transcription initiation capability of a promoter. Enhancers are relatively orientation and position independent having been found 5' (Laimins, L. et al., Proc.Natl.Acad.Sci. 78: 993 [1981]) and 3' (Lusky, M.L., et al., Mol. Cell Bio. 3: 1108 [1983]) to the transcription unit, within an intron (Banerji, J.L. et al., Cell 33: 729 [1983]) as well as within the coding sequence itself (Osborne, T.F., et al., Mol. Cell Bio. 4: 1293 [1984]).

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Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the adhesion.

Expression vector systems generally will contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented medium. Two examples are: CHO DHFR<sup>-</sup> cells and mouse LTK<sup>-</sup> cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented medium. An alternative to supplementing the medium is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the

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DHFR or TK gene will not be capable of survival in non supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982), mycophenolic acid, Mulligan, R.C. and Berg, P. Science 209: 1422 (1980) or hygromycin, Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

"Amplification" refers to the increase or replication of an isolated region within a cell's chromosomal DNA. Amplification is achieved using a selection agent e.g. methotrexate (MTX) which inactivates DHFR. Amplification or the making of successive copies of the DHFR gene results in greater amounts of DHFR being produced in the face of greater amounts of MTX. Amplification pressure is applied notwithstanding the presence of endogenous DHFR, by adding ever greater amounts of MTX to the media. Amplification of a desired gene can be achieved by cotransfecting a mammalian host cell with a plasmid having a DNA encoding a desired protein and the DHFR or amplification gene permitting cointegration. One ensures that the cell requires more DHFR, which requirement is met by replication of the selection gene, by selecting only for cells that can grow in the presence of ever-greater MTX concentration. So long as the gene encoding a desired heterologous protein has cointegrated with the selection gene replication of this gene gives rise to replication of the gene encoding the desired protein. The result is that increased copies of the gene, i.e. an amplified

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gene, encoding the desired heterologous protein express more of the desired heterologous protein.

Preferred host cells for expressing the CD antigen variants of this invention are mammalian cell lines, examples including: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, Graham, F.L. et al., J. Gen Virol. 36: 59 [1977] and 293S cells [293 subclones selected for better suspension growth]); baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin, Proc.Natl.Acad.Sci. (USA) 77: 4216, [1980]); mouse sertoli cells (TM4, Mather, J.P., Biol. Reprod. 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51 cells); and TRI cells (Mather, J.P. et al., Annals N.Y. Acad. Sci. 383: 44-68 [1982]).

20

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. One suitable for transformation of the host cells is the method of Graham, F. and van der Eb, A., Virology, 52: 456-457 (1973). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell walls are used as hosts, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F.N. et al., Proc. Natl. Acad. Sci. (USA), 69: 2110 (1972).

30

Construction of suitable vectors containing the desired coding and control sequences employ standard and manipulative ligation techniques. Isolated plasmids or DNA fragments are

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cleaved, tailored, and religated in the form desired to form the plasmids required. Suitable procedures are well known for the construction described herein. See, for example, (Maniatis, T. et al., Molecular Cloning, 133-134 Cold Spring Harbor, [1982];  
5 "Current Protocols in Molecular Biology", edited by Ausubel et al., [1987], pub. by Greene Publishing Associates & Wiley-Interscience).

Correct plasmid sequences are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with ligation mixtures, successful  
10 transformants selected by ampicillin or tetracycline resistance where appropriate, plasmids from the transformants prepared, and then analyzed by restriction enzyme digestion and/or sequenced by the method of Messing et al., *Nucleic Acids Res.* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499  
15 (1980).

Host cells are transformed with the expression vectors of this invention. Thereafter they are cultured in appropriate culture media, e.g. containing substances for inducing promoters,  
20 selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

25 The secreted adhesion variants are recovered and purified from the culture supernatants or lysates of recombinant hosts. Typically, the supernatants are concentrated by ultrafiltration, contacted with a ligand affinity or immunoaffinity matrix so as to adsorb the adhesion variant, and eluted from the matrix.  
30 Optionally, the adhesion is purified by ion exchange chromatography.

Surprisingly, purification of soluble CD4 adhesion from culture medium was unexpectedly difficult. Notwithstanding that the hydrophobic transmembrane region of the antigen had been  
35 deleted, the antigen exhibited a strong tendency to form aggregates

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that could be readily removed from suspension by centrifugation at 1000 x g, and which avidly coat surfaces such as ultrafiltration membranes. This appears to result from the reduction in concentration of albumin or other serum protein (ordinarily present in the crude preparation) to a particular level, below which the truncated antigen no longer remains soluble. This phenomenon appears to be aggravated by exposure of the CD4 adheson to low pH (< about pH 4). As a result, separation procedures (particularly those that employ acid elution, such as immunoaffinity) should be modified so that the eluate is maintained at, or immediately returned to, about neutrality. Further, a surfactant, e.g. a detergent such as Tween 80, should be included with the antigen during the separation procedure. The final purified product will be stabilized with a predetermined protein such as albumin, and/or a detergent.

The purified adheson is formulated into conventional pharmacologically acceptable excipients.

It is administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of soluble CD4 adheson/ml plasma. For CD4 adheson variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma will be initially evaluated clinically in order to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor. The ordinary dosage of soluble CD4 is 100 µg/kg of patient weight/day.

The therapeutic CD4 variants are employed with other therapies and agents for the treatment of AIDS, including AZT, neutralizing antibodies and immunocytotoxins, gp120 fragments and vaccines.

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In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

5           "Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent  
10 plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

          "Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in  
15 the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in  
20 about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation  
25 times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

30           "Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section  
35 containing the desired fragment, and separation of the gel from

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DNA. This procedure is known generally (Lawn, R. et al., *Nucleic Acids Res.* 9: 6103-6114 [1981], and Goeddel, D. et al., *Nucleic Acids Res.* 8: 4057 [1980]).

5           "Dephosphorylation" refers to the removal of the terminal  
5' phosphates by treatment with bacterial alkaline phosphatase  
(BAP). This procedure prevents the two restriction cleaved ends of  
a DNA fragment from "circularizing" or forming a closed loop that  
would impede insertion of another DNA fragment at the restriction  
10       site. Procedures and reagents for dephosphorylation and other  
recombinant manipulations are conventional. Reactions using BAP  
are carried out in 50mM Tris at 68°C to suppress the activity of  
any exonucleases which may be present in the enzyme preparations.  
Reactions were run for 1 hour. Following the reaction the DNA  
15       fragment is gel purified.

          "Ligation" refers to the process of forming phosphodiester  
bonds between two double stranded nucleic acid fragments (Maniatis,  
T. et al., *Id.* at 146). Unless otherwise provided, ligation may be  
20       accomplished using known buffers and conditions with 10 units of T4  
DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts  
of the DNA fragments to be ligated.

          "Filling" or "blunting" refers to the procedures by which  
25       the single stranded end in the cohesive terminus of a restriction  
enzyme-cleaved nucleic acid is converted to a double strand. This  
eliminates the cohesive terminus and forms a blunt end. This  
process is a versatile tool for converting a restriction cut end  
that may be cohesive with the ends created by only one or a few  
30       other restriction enzymes into a terminus compatible with any  
blunt-cutting restriction endonuclease or other filled cohesive  
terminus. Typically, blunting is accomplished by incubating 2-  
15µg of the target DNA in 10mM MgCl<sub>2</sub>, 1mM dithiothreitol, 50mM  
NaCl, 10mM Tris (pH 7.5) buffer at about 37°C in the presence of 8  
35       units of the Klenow fragment of DNA polymerase I and 250 µM of each



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of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. phenol and chloroform extraction and ethanol precipitation.

5           The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

#### Example 1

#### 10           Construction of Vectors for the Expression of Native CD4 and Secreted Derivatives

##### Section 1

The plasmid used for recombinant synthesis of human CD4 was pSVeCD4DHFR. The plasmid was constructed as follows:

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λCD4P1 containing most of the coding sequence of human CD4 (obtained from a human placental cDNA library using oligonucleotide probes based on the published sequence [Maddon et al. 1985]) was digested with EcoRI to produce the cDNA insert. This fragment was recovered by polyacrylamide gel electrophoresis (fragment 1).

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pUC18 was digested with EcoRI and the single fragment recovered by polyacrylamide gel electrophoresis (fragment 2). Fragment 1 was ligated to fragment 2 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct DNA fragments. This plasmid is referred to as pUCCD4.

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pSVeE'DHFR (Muesing et al., Cell 48:691-701 [1987]) was digested with KpnI and BamHI and blunted with *E. coli* DNA polymerase I (Klenow fragment) and the four dNTPs. Fragment 3 containing the pML-Amp<sup>r</sup> region, SV40 early promoter, the HIV LTR, and the mouse DHFR gene was recovered by gel electrophoresis,

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ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the BamHI restriction site and the absence of the KpnI restriction site. This plasmid is referred to as pSVeABKDHFR and allows EcoRI-BamHI fragments to be inserted after the SV40 early promoter and transcribed under its control, following transfection into an appropriate cell line.

Synthetic oligonucleotides (adaptors 1-8, below) were made to extend from 76 bp 5' of the initiation codon of CD4 translation to the RsaI restriction site at 121 bp 3' of the initiator, with the sequence AATT at the 5' end of the sense strand to generate an end which could ligate to an EcoRI restriction fragment. These oligonucleotides were ligated and the 204 bp fragment containing the entire sequence recovered by gel electrophoresis (fragment 4).

CD4 adaptor 1: AATTCAAGCCCAGAGCCCTGCCATTTCTGTGGGCTCAGGTCCCT  
 CD4 adaptor 2: pACTGCTCAGCCCCCTTCCTCCCTCGGCAAGGCCACAATGAACCGGGGAGTC  
 CD4 adaptor 3: pCCTTTTAGGCACTTGCTTCTGGTGCTGCAACTGGCGCTCCTCCCAGC  
 CD4 adaptor 4:  
 pAGCCACTCAGGGAAACAAAGTGGTGCTGGGCAAAAAAGGGGATACAGTGGAACCTGACCTGT  
 CD4 adaptor 5: pACAGGTCAGTTCCACTGTATCCCCTTTTTTGGCCAGCACCACCTTTGTTTCC  
 CD4 adaptor 6: pCTGAGTGGCTGCTGGGAGGAGCGCCAGTTGCAGCACCAGAAGCAAGT  
 CD4 adaptor 7: pGCCTAAAAGGGACTCCCCGGTTCATTGTGGCCTTGCCGAGGGAGGAAGGG  
 CD4 adaptor 8: GCTGAGCAGTAGGGACCTGAGCCACAGAAATGGCAGGGCTCTGGGCTTG

pUCCD4 was digested with RsaI and SstI and the 401 bp fragment containing part of the CD4 coding sequence recovered by gel electrophoresis (fragment 5). pUC18 was digested with EcoRI and SstI and the fragment comprising the bulk of the plasmid recovered by gel electrophoresis (fragment 6). Fragments 4 and 5 were ligated to fragment 6 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on

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ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of the inserted synthetic DNA was checked by excising the 605 bp EcoRI-SstI fragments from several transformants and ligating them to M13mp19 which had been digested with the same enzymes. After transformation into *E. coli* strain JM101, single-stranded DNA was prepared and sequenced. One plasmid which contained the correct sequence was selected, and is referred to as pCD4int.

pCD4int was digested with EcoRI and SstI and fragment 7 containing the 5' end of the CD4 coding region was recovered by gel electrophoresis. pUCCD4 was digested with SstI and BamHI and the 1139 bp fragment containing the remainder of the CD4 coding region (fragment 8) recovered by gel electrophoresis.

pSV $\Delta$ BKDHFR was digested with EcoRI and BamHI and fragment 9 comprising the bulk of the plasmid was isolated. Fragments 7, 8 and 9 were ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and the resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pSV $\Delta$ CD4DHFR, and was used to direct synthesis of recombinant intact CD4.

## Section 2

A plasmid was constructed to direct the synthesis of a CD4 derivative lacking the putative transmembrane domain and most of the putative cytoplasmic domain (Maddon et al.). This was done with the intention of creating a secreted form of CD4, based on the assumption that these domains anchor the CD4 glycoprotein to the cell membrane, and that their deletion would result in the secretion of the product. This plasmid is referred to as pSV $\Delta$ CD4 $\Delta$ N1 $\Delta$ DHFR and was constructed as follows:

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pUCCD4 was digested with SstI and TaqI and the 531 bp fragment (fragment 10) recovered. pUCCD4 was digested with NlaIII and TaqI and the 112 bp fragment (fragment 11) recovered. pUCCD4 was digested with BamHI and NlaIII and the 301 bp fragment (fragment 12) recovered. pCD4int was digested with SstI and BamHI and fragment 13 comprising the bulk of the plasmid recovered. Fragments 10, 11, and 12 were ligated together with fragment 13 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. Plasmid DNA from several transformants was sequenced to ensure that the 195 bp NlaIII fragment had been deleted and that the proper reading frame was restored. The resulting plasmid is referred to as pCD4ΔNla.

pCD4ΔNla was digested with EcoRI and BamHI and the 1541 bp fragment containing the sequence of a CD4 derivative lacking the transmembrane and cytoplasmic domains recovered (fragment 14) and ligated to fragment 9 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pSVeCD4ΔNlaDHFR.

Both pSVeCD4DHFR and pSVeCD4ΔNlaDHFR were transfected into CHO cells by the same method used to establish cell lines stably expressing HIV-I polypeptides (Muesing, Smith and Capon, Cell 48:6910701 [1987]). These cells were assayed for production by radioimmunoprecipitation as described below. While no product was detected in initial experiments, subsequent experiments showed that the above described coding segment could indeed direct the

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synthesis of a soluble CD4 adhesion variant both in CHO and 293 cells.

### Section 3

5           A different expression system was initially used for the synthesis and expression of a CD4 variant lacking completely the cytoplasmic and transmembrane domains. This system uses the cytomegalovirus promoter and can be used in cultured cells of human origin. The first plasmid constructed for use in this system  
10 contained the entire coding region for CD4 and was intended to function as a control in the following studies. It is referred to as pRKCD4, and was constructed as follows:

          pSVeCD4DHFR was digested with EcoRI and BamHI and fragment  
15 15 containing the entire CD4 coding region was isolated. pRK5 (U.S.S.N. 97,472, filed Sept. 11, 1987) was digested with EcoRI and BamHI and fragment 16 comprising the bulk of the plasmid recovered by gel electrophoresis, ligated to fragment 15, and the ligation mixture transformed into *E. coli* strain 294. The transformed  
20 culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4.

### 25           Section 4

          The next plasmid constructed was designed to direct the expression of the above-mentioned (Section 3) secreted derivative of CD4. The coding region of CD4 was fused after amino acid residue 368 of mature CD4 to a sequence from pBR322 which codes for  
30 9 more residues before a translation termination codon. This removes the putative CD4 transmembrane and cytoplasmic domains, which are presumed to anchor CD4 to the cell surface. The plasmid is referred to as pRKCD4T (and which produces protein called CD4T), and was constructed as follows:

35

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pSVeCD4DHFR was digested with HpaII, blunted with Klenow fragment and the four dNTPs, and digested with BstEII. The 382 bp fragment (fragment 17) containing part of the CD4 coding sequence was recovered by gel electrophoresis. pSVeCD4DHFR was digested with EcoRI and BstEII and the 874 bp fragment (fragment 18) recovered. pBR322 was digested with HindIII, blunted with Klenow fragment and the four dNTPs, and digested with EcoRI. Fragment 19 comprising the bulk of the plasmid was isolated and ligated to fragments 17 and 18 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pCD4Tint.

pRK5 was digested with EcoRI and SmaI and fragment 20 comprising the bulk of the plasmid isolated. pCD4Tint was digested with EcoRI and EcoRV and the 1410 bp fragment containing the CD4 coding sequence to the HpaII site at 1176 bp 3' of the initiating codon and the 154 bp HindIII-EcoRV fragment of pBR322 was recovered (fragment 21). Fragments 20 and 21 were ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4T.

#### Section 5a

In order to create a secreted form of CD4 which could be purified with an antibody directed to herpes virus type I glycoprotein D, a plasmid was constructed to express a derivative of CD4T in which the region coding for the mature, processed CD4T polypeptide was fused to a sequence coding for the signal peptide and the first 27 residues of the mature type I Herpes Simplex Virus

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gD glycoprotein. This plasmid is referred to as pRKGD4T, and was constructed as follows:

5 pgDTrunc.DHFR was digested with EcoRI and PvuII and the fragment containing the coding region for the signal peptide and first 27 residues of the mature HSV I gD glycoprotein was isolated (fragment 22). pRKCD4T was digested with EcoRI and BstEII and fragment 23 containing the 3' end of the CD4 coding sequence and the pRK5 region was isolated.

10 Synthetic oligonucleotides GD (adaptors 1-2, below) containing the coding sequence of CD4 from the codon for the amino terminal residue of mature CD4 to the Rsa site at 121 bp 3' of translation initiation, and containing the sequence CTGCTCGAG at  
15 the 5' end of the sense strand were prepared (fragment 24). pRKCD4 was digested with RsaI and BstEII and the 665 bp fragment containing part of the coding region for CD4 was recovered (fragment 25) and ligated to fragment 24. After digestion with BstEII to ensure that only monomeric fragment was present, the 724  
20 bp fragment containing both sequences was recovered by gel electrophoresis (fragment 26).

25 Fragments 22, 23 and 26 were ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of several transformants was checked to ensure that the synthetic insert was correct and that reading frame  
30 was preserved. This plasmid is referred to as pRKGD4T.

35 These pRK5 derived plasmids preferably were transfected into 293S cells for stable expression according to Muesing, et al. Cell 48:691 (1987) with the exception that in addition to the plasmid of interest a plasmid expressing the neomycin resistance

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gene pRSV neo (Gorman et al. Science 221:553-555 (1985)) was cotransfected. 293 cells also are used satisfactorily as host cells. 2 days after transfection, the cells were passaged into standard medium (1:1 F12/DME supplemented with L-glutamine, penicillin-streptomycin and 10% FBS) with 0.5 mg/ml G418 (Genticin sulfate; Gibco) for selection of stable cell lines, rather than in media containing methotrexate as shown by Muesing et al. Cells were assayed for production of CD4 or CD4 analogs by radioimmuno-precipitation. Binding studies (section 5c) used conditioned supernatants from these cells in the 1:1 F12/DME medium. Materials used in infectivity assays (section 5b) were obtained as described in section 8 below.

gDCD4 adaptor 1:

CTGCTCGAGCAGGGAACAAAGTGGTCTGGGCAAAAAGGGGATACAGTGGAAGTAC

gDCD4 adaptor 2:

PACAGGTCAGTTCCACTGTATCCCTTTTTTGGCCAGCAGCACTTTGTTTCCCTGCTCGA

#### Section 5b

The following constitutes a study of the neutralization of HIV-1 infectivity by soluble CD4 analogs. A modification of the neutralization procedure of Robert-Guroff et al., Nature 316:72 (1985) was followed. Equal volumes of inhibitor supernatant and virus (60 microliters) were incubated at 4 degrees C for 1 hour, then the same volume of H9 (Gallo et al., Science 224:500, 1984) at  $5 \times 10^6$ /ml was added and incubation continued for 1 hour at 37 degrees C. Following absorption,  $2.5 \times 10^5$  cells in 150 microliters were transferred to 2 ml of incubation media. After 4 days at 37 degrees C, the cultures were split 1:2 with fresh media and incubated for an additional 3 days. Cultures were harvested, reverse transcriptase activity was measured (Groopman et al., AIDS Research and Human Retroviruses 3:71, 1987), and immunofluorescence reactivity with HIV-1 positive serum was determined as described (Poiesz et al., Proc. Acad. Nat. Sci. USA 77:7415, 1980).



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Inhibitor supernatants were obtained from confluent plate cultures of 293S/CDT4, 293S/gDCD4T cells or untransfected 293S cells by replacing the growth medium incubation media and harvesting the supernatants 24 hours later. Inhibitor supernatant replaced part or all of the incubation media during the first three days of culture as indicated in the second column of Table 3. Challenge dose of virus was 100 TCID<sub>50</sub> (Groopman et al., supra) of HIV-1 strain HTLV-IIIB grown in H9 cells assayed in the same system. Incubation media consisted of RPMI 1640 media containing 2mM L-glutamine, 100 units/ml penicillin, 100 micrograms/ml streptomycin, 2 micrograms/ml polybrene and 20% fetal calf serum (M.A. Bioproducts).

Table 3

Inhibitor supernatant	Dilution of Inhibitor supernatant	Indirect immunofluorescence (% positive cells)		Reverse transcriptase (cpm/ml x 10 <sup>5</sup> )	
mock-trans- fected	undil.; 1:4	65.3	65.5	21.8	23.9
mock-trans- fected	undil.; 1:4	61.2	61.1	18.5	28.1
CD4T	undil.; 1:4	0.4	18.0	0.11	5.94
CD4T	undil.; 1:4	0.8	16.1	0.15	3.72
gDCD4T	undil.; 1:4	0.4	26.8	0.14	9.92
gDCD4T	undil.; 1:4	1.4	36.1	0.23	11.3

Both forms of soluble CD4 virtually abolished the growth of HIV-1, when incubated with virus-infected cells without prior dilution (Table 2). At a dilution of 1:4 the soluble CD4 preparations were only partially effective in inhibiting virus growth, however the level of fluorescent-positive cells and reverse transcriptase was still significantly lower than cultures receiving mock-transfected cell supernatants (Table 2). Since there was no significant difference in virus growth between diluted and

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undiluted control supernatants, nor did any of the supernatants affect the growth of uninfected H9 cells (data not shown), soluble CD4 proteins present in these supernatants were concluded to be responsible for the neutralization of HIV-1 infection of H9 cells.

5

### Section 5c

To determine the affinity constant for interactions between gp120 and CD4 or CD4 variants, saturation binding analysis was carried out with soluble CD4 (supra) and detergent solubilized intact CD4 (Lasky et al. Cell 50:975 [1987]) employing radioiodinated gp120 labeled with lactoperoxidase. Binding reactions consisted of  $^{125}\text{I}$ -gp120 (3 ng to 670 ng, 2.9 nCi/ng) incubated for 1 hour at 0 degrees C with cell lysates containing intact CD4 (Laskey et al., op cit.) or cell supernatants containing unlabeled CD4T or gDCD4T prepared as described in section 5a. Reactions (0.2ml) had a final composition of 0.5X McDougal Lysis Buffer (McDLB) (1 x McDLB contains 0.5 % Nonidet NP-40, 0.2% Na deoxycholate, 0.12 M NaCl, 0.02 M Tris-HCl, pH 8.0) and were performed in duplicate, both in the presence or absence of 50 micrograms of unlabeled purified gp120 (74 fold or greater excess). Following incubation, bound gp120 was quantitated by immunoprecipitation and counted in a gamma counter. For immunoprecipitation, binding reaction solutions were preabsorbed with 5 microliters of normal rabbit serum for one hour at 0°C, and cleared with 40 microliters of Pansorbin (10 % w/v, Calbiochem) for 30 minutes at 0 degrees C. Samples were then incubated overnight at 0 degrees C with 2 microliters of normal serum or 5 microliters (0.25 microgram) of OKT4 monoclonal antibody (Ortho) followed by collection of immune complexes with 10 microliters of Pansorbin. Precipitates were washed twice in 1X McDLB and once in water, then eluted by eluting at 100 degrees C for 2 minutes in sample buffer (0.12 M Tris-HCl pH 6.8, 4% SDS, 0.7 M mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue). CD4 molecules were bound saturably by gp120, and yielded a simple mass action binding curve.

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Supernatants from mock-transfected cells gave a level of specifically bound gp120 less than 1% that found for supernatants containing soluble CD4. Scatchard analysis revealed a single class of binding sites on each molecule, with apparent dissociation constants ( $K_d$ ) of  $1.3 \times 10^{-9}$  M,  $0.83 \times 10^{-9}$  M and  $0.72 \times 10^{-9}$  M for intact CD4, CD4T and gDCD4T, respectively. The values obtained for CD4-gp120 binding in solution are comparable to the affinity previously measured for gp120 binding to CD4 on whole cells ( $K_d=4.0 \times 10^{-9}$  M. Lasky, Cell, *supra*).

#### Section 6

In order to produce secreted derivatives of CD4 which are free of extraneous amino acid residues, two plasmids were constructed for expression in 293 cells. The plasmids contain CD4 genes which have been truncated without the addition of extra residues, and are referred to as pRKCD4 $\Delta$ N1a and pRKCD4TP (and which produce proteins called CD4TP and CD4 $\Delta$ N1a), and were constructed as follows:

Fragment 14 containing the CD4 gene with the 195 bp NlaIII restriction fragment deleted was ligated to fragment 16, which is pRK5 digested with EcoRI and BamHI. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4 $\Delta$ N1a.

Synthetic DNA (5'CGT GAT AGA AGC TTT CTA GAG 3') was made to attach to the HpaII site at 1176bp and which when so attached would terminate translation after amino acid residue 368 of mature CD4 (fragment 27). The other end of this fragment was designed to ligate to BamHI restriction fragments. pUCCD4 was digested with BstEII and HpaII and the 382bp fragment containing part of the CD4 gene was recovered (fragment 28). Fragments 27 and 28 were ligated

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and then digested with BstEII to reduce dimerized fragments to monomers, and the resulting 401bp fragment was recovered (fragment 29).

5           pRKCD4 was digested with BstII and BamHI and the fragment comprising the bulk of the plasmid (fragment 30) was isolated and ligated to fragment 29. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was  
10       prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4TP. Both plasmids are transfected into 293 cells to generate stable variant CD4-expressing cell lines as described above.

15

#### Section 7

Two plasmids were constructed to direct the expression of secreted CD4 lacking extraneous amino acid residues in CHO cells.  
20       These are referred to as pSVeCD4 $\Delta$ N1aSVDHFR and pSVeCD4TPSVDHFR (and which encode proteins having the primary sequence of CD4 $\Delta$ N1a and CD4TP), and were constructed as follows:

25           pE348HBV.E400D22 was digested with PvuI and EcoRI and the fragment containing the SV40 early promoter and part of the  $\beta$ -lactamase gene was recovered (fragment 31). pE348HBV.E400D22 was digested with PvuI and BamHI and the large fragment containing the balance of the  $\beta$ -lactamase gene as well as the SV40 early promoter and the DHFR gene was isolated (fragment 32).

30

Fragments 31 and 32 were ligated together with fragment 14 and transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked  
35       by restriction analysis for the presence of the correct fragment.

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The resulting plasmid is referred to as pSVECD4 $\Delta$ NlaSVDHFR. This plasmid contains the same DNA fragment encoding the soluble CD4 molecule found in the above-mentioned plasmid pSVeCD4 $\Delta$ NlaDHFR (Section 2).

5

pRKCD4TP was digested with EcoRI and BamHI and the fragment containing the truncated CD4 coding region was isolated and ligated to fragments 31 and 32. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pSVeCD4TPSVDHFR. Both of these plasmids are transfected into CHO cells and amplified transfectants selected by methotrexate using conventional procedures.

15

### Example 2

Fusions of the V region of the CD4 gene, which is homologous to the variable region of immunoglobulin genes (ref Maddon et al. 1985), to the constant (C) region of human immunoglobulin  $\kappa$  and  $\gamma 2$  chains are constructed as follows:

20

Synthetic DNA is made to code for the C region of human  $\kappa$  chain (residues 109-214) based on the sequence published by Morin et al., Proc. Natl. Acad. Sci. 82:7025-7029, with the addition at the 5' end of the coding strand of the sequence GGGG, which allows this fragment to be ligated to the BspMI site at the end of the putative V-like region of CD4. At the 3' end of the coding region, a translational stop codon is added as well as a sequence which allows this end to be ligated to BamHI restriction fragments. The synthetic DNA is made in 8 fragments, 4 for each strand, 70-90 bases long. These are then allowed to anneal and ligated prior to isolation on a polyacrylamide gel (fragment 33).

30

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pRKCD4 is digested with EcoRI and BspMI and the 478bp fragment containing the region coding for the putative V-like domain of CD4 is recovered (fragment 34). Fragments 33 and 34 are ligated together with fragment 16 (from the expression vector pRK5). The ligation mixture is transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA is prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4Ck.

A plasmid encoding a fusion of the CD4 V-like domain to the human immunoglobulin C $\gamma$ 2 region is constructed in a similar fashion, and is referred to as pRKCD4C $\gamma$ 2. Both of these plasmids are transfected into 293 cells, myeloma cells or other competent cells in order to obtain cell lines expressing variant CD4 molecules as described above.

### Example 3

The gDCD4T secreted by the method of Example 1 was purified from cell culture fluid containing either 10% FBS (fetal bovine serum) or no added FBS. The conditioned cell culture fluid was first concentrated by ultrafiltration then purified by immunoaffinity chromatography. The immunoaffinity column was produced by coupling murine monoclonal antibody 5B6 (whose epitope is on the HSV-1 gD portion of the gDCD4T molecule) to glyceryl coated controlled pore glass by the method of Roy *et al.*, 1984. The concentrated cell culture fluid is applied directly to the column and the contaminating proteins are washed away with neutral pH buffer. The column is then washed with neutral buffer containing tetramethylammonium chloride followed by neutral buffer containing Tween 80. The bound gDCD4T is eluted from the column with buffer at pH3 containing Tween 80 (0.1% w/v) and is neutralized immediately as it is eluted. The eluted neutralized

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gDCD4T is then concentrated by ultrafiltration and dialyzed/diafiltered to exchange the buffer for a physiological salt solution containing Tween 80 at approximately 0.1% w/v.

5           If the detergent is not present the gDCD4T forms aggregates as evidenced by the ability of centrifugation at approximately 10,000 Xg for 2 minutes to remove the gDCD4T from the solution. Incubation of gDCD4T at 4°C in 0.1M sodium acetate, 0.5M NaCl and 0.25M tris at pH 7 together with BSA, Tween 80 or glycerol as  
10 candidate stabilizers showed that, in the absence of a stabilizer the gDCD4T gradually aggregated over the space of 12 days to the point where only about 60-70% of the protein was soluble. However, use of 0.1% w/v Tween 80 or (0.5 mg/ml BSA ensured that about 100% or 80%, respectively, of the gDCD4T remained soluble over this  
15 period. Surprisingly glycerol was ineffective as a stabilizer and produced results inferior even to the control-at 8 days about 80% of the gDCD4T was aggregated when stored in the presence of glycerol.

20

#### Example 4

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin  $\gamma 1$ . These plasmids are referred to as pRKCD4<sub>2</sub> $\gamma 1$ ,  
25 pRKCD4<sub>e4</sub> $\gamma 1$ , pRKCD4<sub>2</sub> $\gamma 1$ , pRKCD4<sub>e2</sub> $\gamma 1$ , pRKCD4<sub>1</sub> $\gamma 1$ , and pRKCD4<sub>e1</sub> $\gamma 1$ .

Plasmid pRKCD4<sub>4</sub> $\gamma 1$  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine reside 366 of the mature CD4 polypeptide, immediately followed by  
30 the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

Plasmid pRKCD4<sub>e4</sub> $\gamma 1$  contains the portion of the CD4 gene  
35 from the initiation codon to the fusion site after the codon for

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lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

5

Plasmid pRKCD4<sub>2</sub> $\gamma 1$  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for glutamine residue 180 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

10

Plasmid pRKCD4<sub>e2</sub> $\gamma 1$  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 177 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

15

Plasmid pRKCD4<sub>1</sub> $\gamma 1$  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for aspartic acid residue 105 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

20

25

Plasmid pRKCD4<sub>e1</sub> $\gamma 1$  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 100 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin  $\gamma 1$ , starting at the codon for serine residue 114 of mature human immunoglobulin  $\gamma 1$  (Kabat et al.).

30



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Construction of these plasmids required the prior construction of plasmid pRKCD4TP/ $\gamma$ 1. It was constructed as follows:

5           A cDNA clone coding for human immunoglobulin  $\gamma$ 1 was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Ellison *et al.*, "Nucl. Acids Res." 10:4071-4079 [1982]), and an EcoRI-EagI fragment (the EcoRI site was contributed by a linker; see Fig. 4a,b) containing part of the variable and all of the constant region was obtained. This fragment was blunted with Klenow fragment, and recovered by gel electrophoresis (Fragment al).

15           Plasmid pRKCD4TP-kk, encoding a substitutional variant of soluble CD4 (residues 1-368) containing a lysine residue instead of asparagine at position 1 of the mature polypeptide, was constructed from plasmid pRKCD4TP by site-directed mutagenesis. A synthetic oligonucleotide was made as a primer for a mutagenesis reaction to obtain the desired coding sequence. This was synthesized as a 51-mer which contained two silent mutations from the natural sequence in addition to the substitution mutation, and 21 bases on each side of the mutated codons:

20           5' - CCC TTT TTT GCC CAG CAC CAC CTT CTT GCC CTG-  
25           AGT GGC TGC TGG GAG GAG -3'

30           Plasmid pRKCD4TP was transformed into *E. coli* strain SR101 and the transformed colonies plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13K07 helper bacteriophage to yield secreted, encapsidated single-stranded templates of pRKCD4TP. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reaction was transformed *E. coli* SR101 and the transformed culture plated on ampicillin media plates.

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Transformants were screened by colony hybridization (ref. Grunstein-Hogness) for the presence of the appropriate sequence, using the following 16 mer as the probe.

5' - C CAG CTT CTT GCC CTG -3'

5 The hybridization conditions chosen were sufficiently stringent that the probe only detects the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of ml3K07 bacteriophage. Templates were prepared as above and screened by sequencing.

15 Plasmid pRKCD4TP-kk was digested with XbaI and treated with Klenow Enzyme, and Fragment a2, containing the linearized plasmid was recovered by gel electrophoresis, and ligated with fragment a1. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from the transformants and checked by restriction analysis for the presence of the correct fragment in the correct orientation (i.e., the immunoglobulin coding region in the same orientation as the CD4 coding region, and at the 3' end of the CD4 coding region). This plasmid is referred to as pRKCD4TP/γ1.

25 Synthetic oligonucleotides were made as primers for deletional mutagenesis reactions to fuse the appropriate coding sequences of IgG1 and CD4 as described above. These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH<sub>2</sub>-terminal 8 residues of the desired immunoglobulin moiety). Plasmid pRKCD4TP/γ1 was transformed into *E. coli* strain SR101 and the transformed cultures plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of ml3K07 helper bacteriophage

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to yield secreted, encapsidated single-stranded templates of pRKCD4TP/ $\gamma$ 1. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed E. coli SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (ref. Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into E. coli strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13K07 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmids were transfected into 293 cells using standard procedures and assayed for expression and production as described above.

	<u>Expressed</u>	<u>Secreted</u>
pRKCD4 <sub>1</sub> $\gamma$ 1	+	-
25 pRKCD4 <sub>e2</sub> $\gamma$ 1	+	+
pRKCD4 <sub>2</sub> $\gamma$ 1	+	+
pRKCD4 <sub>e4</sub> $\gamma$ 1	+	+
pRKCD4 <sub>4</sub> $\gamma$ 1	+	+

Plasmids also were constructed to direct the expression of fusion proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the truncated portion of the constant region of human immunoglobulin  $\gamma$ 1, comprising only the hinge region and constant domains CH<sub>2</sub> and CH<sub>3</sub>.

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Synthetic oligonucleotides were made as primers for mutagenesis reactions to delete the immunoglobulin sequence from Ser114 to Cys215 inclusive (Kabat et al.). These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH<sub>2</sub>-terminal 8 residues of the desired immunoglobulin moiety). Plasmids pRKCD4<sub>4</sub>γ1, pRKCD4<sub>2</sub>γ1 and pRKCD4<sub>1</sub>γ1 were separately transformed into E. coli strain SR101 and the transformed culture plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13K07 helper bacteriophage to yield secreted, encapsidated single-stranded templates of these plasmids. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed E. coli SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into E. coli strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13K07 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmid derived from plasmid pRKCD4<sub>4</sub>γ1 is referred to as pRKCD4<sub>4</sub>Fc1, that derived from plasmid pRKCD4<sub>2</sub>γ1 is referred to as pRKCD4<sub>2</sub>Fc1 and that derived from plasmid pRKCD4<sub>1</sub>γ1 is referred to as pRKCD4<sub>1</sub>Fc1.

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pRKCD4<sub>2</sub>Fc1, pRKCD4<sub>1</sub>Fc1 and pRKCD4<sub>4</sub>Fc1 are cultured in the same fashion as described above and CH1-deleted CD4 immunoadhesons recovered as described elsewhere herein.

5     Light Chain Fusions

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin  $\kappa$ . These plasmids are referred to as pRKCD4<sub>4 $\kappa$</sub>  and  
10     pRKCD4<sub>e4 $\kappa$</sub> .

Plasmid pRKCD4<sub>4 $\kappa$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by  
15     the sequence for the constant region of human immunoglobulin  $\kappa$ , starting at the codon for threonine residue 109 of the mature human immunoglobulin  $\kappa$ . (Kabat et al.)

Plasmid pRKCD4<sub>e4 $\kappa$</sub>  contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by  
20     the sequence for the constant region of human immunoglobulin  $\kappa$ , starting at the codon for threonine residue 109 of the mature human immunoglobulin  $\kappa$ . (Kabat et al.)

25     These plasmids were constructed in a manner analogous to plasmids pRKCD4<sub>4 $\gamma$ 1</sub> and pRKCD4<sub>e4 $\gamma$ 1</sub> described above, with the following exception:

30     The human immunoglobulin  $\kappa$  coding sequence (Fig. 5) was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Hieter, P.A. et al., Cell 22:197-207 [1980]) and an EcoRI-BspMI fragment containing part of the variable region and the entire  
35     constant region was obtained (see Fig. 5). This fragment was

-51-

blunted with Klenow fragment and the four dNTPs. This fragment was used instead of fragment a1, and was used to construct plasmid pRKCD4TP/hκ.

5     Expression in CHO Cells

Plasmids were or are constructed to direct the expression of the immunoadhesons described above in CHO cells. These are referred to as pSVeCD4<sub>4γ1</sub>SVDHFR, pSVeCD4<sub>2γ1</sub>SVDHFR, pSVeCD4<sub>1γ1</sub>SVDHFR, pSVeCD4<sub>e4γ1</sub>SVDHFR, pSVeCD4<sub>e2γ1</sub>SVDHFR, 10 pSVeCD4<sub>e1γ1</sub>SVDHFR, pSVeCD4<sub>4Fc1</sub>SVDHFR, pSVeCD4<sub>2Fc1</sub>SVDHFR, pSVeCD4<sub>1Fc1</sub>SVDHFR, pSVeCD4<sub>4κ</sub>SVDHFR and pSVeCD4<sub>2κ</sub>SVDHFR.

Fragment 31 was prepared as described above. Fragment 32a was prepared by digesting plasmid pE348HBV.E400 D22 with BamHI, 15 blunting with Klenow fragment and the four dNTPs, then digesting with PvuI and isolating the large fragment containing the balance of the β-lactamase gene and the SV40 early promoter and the DHFR gene. Plasmids pRKCD4<sub>4γ1</sub>, pRKCD4<sub>2γ1</sub>, pRKCD4<sub>1γ1</sub>, pRKCD4<sub>e4γ1</sub>, pRKCD4<sub>e2γ1</sub>, pRKCD4<sub>e1γ1</sub>, pRKCD4<sub>4Fc1</sub>, pRKCD4<sub>2Fc1</sub>, pRKCD4<sub>1Fc1</sub>, 20 pRKCD4<sub>4κ</sub> and pRKCD4<sub>2κ</sub> were separately digested with HindIII, blunted with Klenow fragment and the four dNTPs, then digested with EcoRI and the fragments encoding the CD4-Ig fusion protein were isolated. The resulting DNA fragments were ligated together with fragments 31 and 32a and transformed into *E. coli* strain 294. 25 Colonies were selected and checked for the presence of the correct plasmid as above, then transfected into CHO cells and amplified by methotrexate selection using conventional procedures.

Example 5

30     Culture, Purification and formulation of CD4 variants

Plasmids encoding soluble CD4 adhesons such as CD4T, CD4TP, or soluble CD4 immunoadhesons were calcium phosphate transfected into CHO-DP7 (a proinsulin-transformed autocrine host cell derived 35 from CHO; U.S.S.N. 97,472) and the transformants grown in selective

-52-

medium (1:1 HAM F12/DMEM GHT<sup>+</sup> containing 1 - 10% diafiltered or dialyzed bovine serum). Other suitable host cells are CHO cells or 293S human embryonic kidney cells. The transformants were amplified by methotrexate selection in the same medium but containing 500 nm methotrexate. A subclone capable of secreting CD4TP, CD4tp 500 b, was selected. CD4tp 500 b is cultured in a DMEM/HAM F12 medium at about 37°C until CD4TP accumulates in the culture, after which the medium is separated from the cells and insoluble matter by centrifuging.

Culture fluid from CD4TP transformants was concentrated and diafiltered to lower the ionic strength. The concentrate was passed through a large volume of Q-Sepharose anion exchange resin (previously equilibrated with 25 mM NaCl, pH 8.5) in order to adsorb contaminants from the culture fluid. The isoelectric point of CD4TP is about 9.5, thus making it possible to discriminate between truncated forms of CD4 and most contaminants by alternate adsorption, respectively, on a cation exchange resin such as carboxymethyl or sulfonyl Sepharose, and an anion exchange resin such as quaternary ammonium Sepharose. In addition, since highly electropositive domains are present in the extracellular segment of CD4 any CD4-containing variant is purified in the same fashion as CD4TP. The unadsorbed culture fluid from the anion exchange resin step was then passed through a cation exchange resin (previously equilibrated with 25 mM NaCl at pH 8.5) whereby CD4TP was adsorbed to the resin. The CD4TP was eluted with a NaCl gradient at pH 8.5, this CD4 variant eluting at about 0.2 M NaCl. Ammonium sulfate was added to the eluate to a concentration of 1.7M and the solution passed through a column of hydrophobic interaction chromatography resin (phenyl or butyl Sepharose). The CD4TP was eluted from the hydrophobic interaction column with a gradient of ammonium sulfate, the CD4TP emerging at about 0.7M ammonium sulfate. The eluate was concentrated and buffer exchanged on a G-25 column using phosphate buffered saline containing .02 % (w/v) Tween 20 or Tween 80. The CD4TP was soluble and stable in this solution, which was sterile

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filtered and filled into vials as an aqueous formulation. Other polymeric nonionic surfactants are suitably used with the CD4 formulations, including Pluronic block copolymers or polyethylene glycol.

5

It is also possible to employ immunoaffinity purification of soluble CD4 wherein the CD4 is adsorbed onto an immobilized antibody against CD4. This method suffers from the disadvantage that elution of the soluble CD4 under acidic conditions leads to protein aggregation that is only thoroughly ameliorated at relatively higher levels of surfactant. The foregoing procedure permits the use of much lower quantities of surfactant, about from 0.01 to 0.10 % (w/v) surfactant.

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The procedure followed for the purification of CD4 fusions with immunoglobulin heavy chain was to concentrate recombinant supernatants by ultrafiltration and thereafter adsorb the fusion onto resin-immobilized Staphylococcal protein A. The fusion was eluted with 0.1M citrate buffer pH 3 with no salt or detergent. This preparation is buffered into Tris buffer at pH 7.5. The immunoglobulin fusions with CD4 V1-V4 optionally are further purified by the procedure described above for unfused CD4 variants. CD4 immunoglobulin fusions with CD4 V1-V2 also may be purified by the procedure above, except that it is not expected that the isoelectric point of this class of molecules will be as alkaline as that of species containing all four V regions of CD4.

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#### Example 6

The characteristics of several adhesion variants were determined. As shown in table 4 the immunoadhesons CD4<sub>4</sub>γ1 and CD4<sub>2</sub>γ1 show improved plasma half-life in rabbits, coupled with high-affinity gp120 binding and an affinity for Fcγ receptor (determined with U937 cells) that is comparable to that of bulk human IgG1.

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Table 4

	gp120 KD (nM) <sup>#</sup>	FcγR KD (nM) <sup>+</sup>	Plasma Half-Life <sup>++</sup> In Rabbits (Hrs.)	
5	CD4T <sup>S</sup>	2.3 ± 0.4	Not detected	0.25.
	CD4 <sub>4</sub> γ1	1.2 ± 0.1	2.83 ± 0.25	6.4
10	CD4 <sub>2</sub> γ1	1.4 ± 0.1	3.01 ± 0.68	40.6
	human IgG1	ND <sup>**</sup>	3.52 ± 0.5	21 days <sup>*</sup>

\* determined in humans

15 + KD was determined by the method of Anderson *et al.*, "J. Immunol." 125:2735-2741 (1980).

# determined by the method of Smith *et al.*, "Science" 238:1704-07 (1987).

§ residues 1-368 only

20 ++ The adheson variant was injected intravenously into rabbits and samples of blood were collected periodically and assayed for the presence of the adheson variant.

\*\* Not done.

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## Claims:

1. Nucleic acid encoding an amino acid sequence variant of an adhesion.
- 5 2. The nucleic acid of claim 1 wherein the adhesion is a CD4 polypeptide.
3. The nucleic acid of claim 2 wherein the variant is a CD4 polypeptide in which nucleic acid encoding the transmembrane domain has been modified whereby the CD4 polypeptide encoded  
10 thereby contains an inactivated transmembrane domain.
4. The nucleic acid of claim 3 wherein the transmembrane domain has been inactivated by its deletion or by substituting for  
15 the transmembrane domain an amino acid sequence having a substantially hydrophilic hydropathy profile.
5. The nucleic acid of claim 2 wherein the variant comprises a fusion of (a) a polypeptide different from the CD4 and (b) a  
20 CD4 polypeptide.
6. The nucleic acid of claim 5 wherein the polypeptide different from the CD4 bears a non-CD4 immune epitope.
- 25 7. The nucleic acid of claim 6 wherein the polypeptide different from CD4 is fused to the amino or carboxyl terminus of mature CD4 and the transmembrane domain of CD4 has been inactivated.
8. The nucleic acid of claim 5 wherein the different polypeptide  
30 comprises a signal sequence.
9. The nucleic acid of claim 5 wherein the different polypeptide contains about from 5 to 1000 residues.

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10. The nucleic acid of claim 9 wherein the different polypeptide is capable of eliciting a humoral immune response in an animal.
- 5 11. The nucleic acid of claim 10 wherein the different polypeptide is a viral polypeptide or an allergen.
12. The nucleic acid of claim 5 wherein the different polypeptide is a human plasma protein having a plasma half life greater  
10 than from which the transmembrane domain has been deleted.
13. The nucleic acid of claim 12 wherein the variant is a fusion of a polypeptide comprising at least one V-like domain of CD4 fused with a polypeptide comprising an immunoglobulin constant  
15 domain.
14. The nucleic acid of claim 1 wherein the adhesion is CD4, CD8 or the high affinity IgE receptor.
- 20 15. The nucleic acid of claim 2 wherein the variant consists essentially of the V<sub>1</sub> through V<sub>4</sub> or V<sub>1</sub> through V<sub>2</sub> regions of the CD4 antigen.
16. The nucleic acid of claim 2 which consists essentially of the  
25 CD4 insert of pCD4ΔN1a.
17. The nucleic acid of claim 12 wherein the different polypeptide is albumin, apolipoprotein or transferrin.
- 30 18. The nucleic acid of claim 8 wherein the signal sequence is a bacterial signal sequence.
19. The nucleic acid of claim 15 wherein the variant consists essentially of CD4 residues 1-368.

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20. The nucleic acid of claim 15 wherein the variant consists essentially of CD4 residues 1-180.
- 5 21. The nucleic acid of claim 13 wherein the immunoglobulin constant domain is the constant domain of an IgG heavy chain.
22. The nucleic acid of claim 5 wherein the different polypeptide is a cytotoxic polypeptide.
- 10 23. The nucleic acid of claim 5 wherein the cytotoxic polypeptide is the diptheria toxin A.
24. A composition comprising an adheson amino acid sequence variant which is incapable of cell membrane anchorage.
- 15 25. The composition of claim 24 wherein the adheson variant comprises a CD4 amino acid sequence capable of binding gp120.
26. The composition of claim 25 further comprising an agent for inhibiting the aggregation of the variant selected from the group of a predetermined protein and a surfactant.
- 20 27. The composition of claim 26 wherein the agent is a surfactant.
- 25 28. The composition of claim 27 wherein the surfactant is Tween 80 or Tween 20.
- 30 29. The composition of claim 25 wherein the CD4 transmembrane domain has been deleted or has been substituted for by an amino acid sequence having a substantially hydrophilic hydropathy profile.
- 35 30. The composition of claim 29 which is sterile and which further comprises a physiologically acceptable carrier.

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31. The composition of claim 25 wherein the variant comprises an immunoglobulin amino acid sequence.
- 5 32. The composition of claim 31 wherein the immunoglobulin sequence comprises a constant domain sequence of an immunoglobulin heavy chain.
- 10 33. The composition of claim 32 wherein the constant domain is linked at its N-terminus to the C-terminus of a transmembrane-deleted CD4 polypeptide.
- 15 34. The composition of claim 33 wherein the CD4 polypeptide contains  $V_1V_2$ .
35. The composition of claim 33 wherein the CD4 polypeptide contains  $V_1V_2V_3V_4$ .
- 20 36. The composition of claim 31 wherein the the variant is in the form of a dimer.
- 25 37. The composition of claim 36 wherein the composition comprises a fusion of a CD4 V-like domain to an immunoglobulin heavy chain constant domain.
- 30 38. The composition of claim 31 wherein the variant is selected from the group consisting of
  - (a)  $AC_L$ ;
  - (b)  $AC_L-AC_L$ ;
  - (c)  $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HCH, V_LCL-AC_H, \text{ or } V_LCL-V_HCH]$ ;
  - (d)  $AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HCH, V_LCL-AC_H, \text{ or } V_LCL-V_HCH]$ ;
  - (e)  $AC_L-V_HCH-[AC_H, AC_L-AC_H, AC_L-V_HCH, V_LCL-AC_H, \text{ or } V_LCL-V_HCH]$ ;
  - (f)  $V_LCL-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HCH, V_LCL-AC_H, \text{ or } V_LCL-V_HCH]$ ;
  - or
  - (g)  $[A-Y]_n-[V_LCL-V_HCH]_2$
- 35

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wherein A is a CD4 polypeptide containing a CD4 variable region-like domain;  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin; n is an integer; and Y designates the residue of a covalent cross-linking agent.

- 5
39. The composition of claim 38 wherein the  $V_L$  and  $V_H$  domains are capable of binding a predetermined antigen.
- 10
40. The composition of claim 31 wherein the immunoglobulin sequence is obtained from IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.
- 15
41. The composition of claim 25 wherein the variant comprises a polypeptide different from CD4 which is nonimmunogenic in humans.
- 20
42. The composition of claim 41 wherein the variant comprises a polypeptide which is immunogenic in humans.
- 25
43. The composition of claim 41 wherein the variant comprises a polypeptide having a human plasma half life which is greater than about 20 hours.
- 30
44. The composition of claim 41 wherein the variant comprises a human transferrin, apolipoprotein or albumin polypeptide.
45. The composition of claim 25 wherein the variant comprises a cytotoxic polypeptide.
- 35
46. The composition of claim 45 wherein the cytotoxic polypeptide is ricin A chain or diphtheria toxin A.
47. A polypeptide comprising a CD4 amino acid sequence capable of binding gp120 which is cross-linked to (a) polypeptide having

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a plasma half life of greater than about 20 hours or (b) a cytotoxic polypeptide.

- 5 48. The polypeptide of claim 47 wherein the polypeptide of (a) is transferrin, an apolipoprotein or albumin.
- 10 49. The polypeptide of claim 47 wherein the cytotoxic polypeptide is cross-linked to the CD4 variable-like domain by a bifunctional cross-linking agent.
50. A method for preparing an adheson variant comprising transfecting a host cell with the nucleic acid of claim 1.
- 15 51. A method for preparing an adheson variant comprising recovering the variant from the culture of a host cell transfected with the nucleic acid of claim 1.
- 20 52. The method of claim 51 wherein the adheson is CD4 and the variant is recovered from the culture medium of the host cell or from the cell itself.
53. The method of claim 52 wherein the variant is recovered by adsorption onto a cation exchange resin.
- 25 54. The method of claim 53 wherein the variant is recovered by adsorption of contaminants onto an anion exchange resin.
55. The method of claim 52 wherein the variant lacks a functional transmembrane domain.
- 30 56. The method of claim 52 wherein wherein a salt is added to the culture medium to occupy charged domains of the variant, the resulting solution is contacted with a hydrophobic affinity chromatography resin to adsorb the variant, and the variant

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eluted from the resin by washing the resin with a declining gradient of salt.

- 5 57. The method of claim 52 wherein the variant is recovered by immunoaffinity chromatography.
- 10 58. The method of claim 57 wherein the immunoaffinity chromatography is directed against a polypeptide different from CD4 which is fused to CD4.
59. A method for the treatment of an HIV infection comprising administering to a patient infected with HIV a therapeutically effective dose of an amino acid sequence variant of CD4.
- 15 60. A replicable vector comprising the nucleic acid of claim 1.



**FIG. 1A**

[illegible]



3/ 13

**FIG. 1B-2**

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[illegible]

**FIG. 1C**

FIG. 2A

FIG. 2A

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## FIG. 2B-1

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scrFI      scrFI      ddeI      pleI      styI      scrFI      bstNI      aluI      bstNI
bspl286    bspI286    mnlI      hinfI      mboII mnlI      pvuII bspI286
601 GAGAGCCCCCTGCTAGTAGCCCTCAGTGCATGTAGGAGTCCAAAGGGGTAACATACAGGGGGGAAGACCCCTCTCCGTGTCTCAGCTGGAGCTCC
CTCTCGGGGGACCATCATCGGGAGTACGTTACATCTCAGGTTCCCATTTTGTATGTCCCTCCCTTCTGGAGAGGACAGATCGACCTCGAGG
176 GluSerProGlySerSerProSerValGlnCysArgSerProArgGlyLysAsnIleGlnGlyGlyThrLeuSerValSerGlnLeuGluLeuGln

scrFI      nlaIV      bspI      mboII      mnlI      haeIII      stuI      haeI
nlaI      bspI      mboII mnlI      nheI      aluI      mnlI
701 AGGATAGTGGCACCTGGACATGCACGTCTTGCAGAACCAAGAGAGGCTGGAGTTCAAAATAGACATCGTGTGCTAGCTTTTCCAGAAGGCCCTCCAGCAT
TCCTATCACCGTGGACCTGTACGTGACAGAACGCTTGTGCTCTTCCACCTCAAGTTTATCTGTAGCACCCAGATCGAAAGGTCTTCCGGAGGTCGTA
210 AspSerGlyThrTrpThrCysThrValLeuGlnAsnGlnLysLysValGluPheLysIleAspIleValLeuAlaPheGlnLysAlaSerSerIle

scrFI      nlaIV      bspI      mboII      mnlI      aluI      aluI      mnlI
nlaI      bspI      mboII mnlI      nheI      aluI      mnlI
801 AGTCTATAAGAAAGAGGGGGAACAGGTGGAGTCTCTCCCTTCCCACTCGCCCTTTACAGTTGAAAAGCTGACGGGAGCTGGCGAGCTGTGGTGGCAGGCGGAG
TCAGATATTCTTCTCCCTTGTCCACCTCAAGAGAGGGTGGAGGGAATGTCAACTTTTCGACTGCCCGCTCACCGCTCGACACCCGCTCCGCCTC
243 ValTyrLysLysGluGlyGluGlnValGluPheSerPheProLeuAlaPheThrValGluLysLeuThrGlySerGlyGluLeuTrpTrpGlnAlaGlu

scrFI      bspI      sau3AI      dpnI      aluI      mboII      bstNI      ecoO      ddeI
mnlI      pflMI      mnlI      aluI      mnlI      bstNI      aluI
901 AGGGCTTCTCCTCCAGTCTTGGATCACCTTTGACCTGAAGAACAGGAAGTGTCTGTAAACGGGTTACCCAGGACCCCTAAGCTCCAGATGGGCAAGA
TCCCGAAGGAGGAGGTTTCAGAACCTAGTGGAACTGGACTTCTTGTCTTCCACAGACATTTTGGCCCAATGGGTCCGATTCGAGGTCTACCCGTTCT
276 ArgAlaSerSerSerLysSerTrpIleThrPheAspLeuLysAsnLysGluValSerValLysArgValThrGlnAspProLysLeuGlnMetGlyLysLys

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          haeIII          sau96I
          stui            scrFI
          haeI            hphI haeIII
                        mnli bstNI
          aluI            mnli ddel
                        mnli
1001 AGCTCCCGCTCCACCTCACCCTGCCCGAGGCTTGCCTCAGTATGCTGGCTCTGGAAACCTCACCCCTGGCCCTTGAGCGGAAACAGGAAAGTTGCATCA
    TCGAGGCGGAGGTGGAGTGGGACGGGTCCGGAACGGAGTCATACGACCGAGACCTTTGGAGTGGACCGGAACTTCGCTTTTGTCTTCAACGTTAGT
310  LeuProLeuHisLeuThrLeuProGlnAlaLeuProGlnTyrAlaGlySerGlyAsnLeuThrLeuAlaLeuGluAlaLysThrGlyLysLeuHisGln

          haeIII          sau96I
          stui            scrFI
          haeI            hphI haeIII
                        mnli bstNI
          aluI            mnli ddel
                        mnli
1101 GGAAGTGAACCTGGTGGTGATGAGAGCCACTCAGCTCCAGAAAATTGACCTGTGAGGTGTGGGACCCACCTCCCTTAAGCTGATGCTGAGTTGAAA
    CCTTCACTTGGACCACTACTCTCGGTGAGTCGAGGTCTTTTAACTGGACACTCCACACCCCTGGTGGAGGGGATTCGACTACGACTCAAACCTTT
343  GluValAsnLeuValValMetArgAlaThrGlnLeuGlnLysAsnLeuThrCysGluValTrpGlyProThrSerProLysLeuMetLeuSerLeuLys

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FIG. 2B-2

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          mnlI      auaI      alwNI      ddeI      hinfI      alwNI
          ddeI      pleI      hinfI      alwNI
          mstII      eco8II      foki      alwNI      ddeI      hinfI      alwNI
          1201 CTGGAGAACAGGAGGCAAGGCTCTCGAAGCGGGAGAGCGGTGGTGGTCTGAACCCCTGAGCGGGGATGTGGCAGTGTCTGCTGAGTCACTCGGGAC
          GACCTCTTGTCTCCGTTCCAGAGCTTCGCCCTCTCCGCCACACCCACGACTTGGGACTCCGCCCTACACCGTCACAGACGACTCACTGAGCCCTG
          376 LeuGluAsnLysGluAlaLysValSerLysArgGluLysAlaValTrpValLeuAsnProGluAlaGlyMetTrpGlnCysLeuLeuSerAspSerGlyGln

          sau96I      aluI      mseI      nlaIV      banI
          avaiI      aluI      mseI      nlaIV      banI
          ppuMI      hinfI      mseI      nlaIV      banI
          1301 AGGTCTGTGGATCCAAACATCAAGTTCTGTGCCACATGGTCCACCCGAGCTTTTAATGCGGTAGTTTATCAGATTAAATGCTAACGCGAGTCAGGCA
          TCCAGGACGACCTTAGTTGTAGTTCCAAGACGGGTGTACCGAGTGGGGCTCGAATTTACGCCNTCAATATAGTGTCAATTTAACGATTGCGTCAGTCCGT
          410 ValLeuLeuGluSerAsnIleLysValLeuProThrTrpSerThrProSerPheAsnAlaValValTyrHisSerOC*

          haeIII      sau96I
          scrFI      nciI      rsaI      mspI      mnlI      hpaII
          1401 CCGTGTATGAATCTAACANTGGCTCATCGTCCGACCGTCAACCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCTCT
          GGCACATACCTTTAGTTTACGCGGAGTAGCAGTAGGAGCCGTGGCAGTGGGACCTACGACATCCGTATCCGAACCAATACGGCCATGACGCCCGGAGAGA

          1501 TCGGGGAT
          ACGCCCTA

```

FIG. 2C



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## CD4

Immunoglobulin  $\gamma_1$ 

## Soluble rCD4

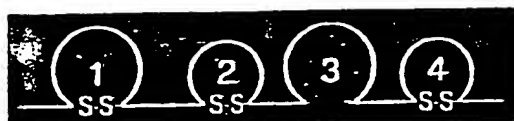
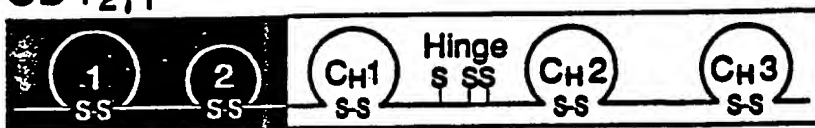
CD4 $_2\gamma_1$ CD4 $_4\gamma_1$ 

FIG. 3

**FIG. 4A**

[illegible]

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**FIG. 4B-1**

[illegible]

FIG 4B-2

**FIG. 4B-2**

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sau96I  
 avaiI  
 nlaIV  
 mnlI styI  
 1 GAATTCACCTCTCACCACATCAGCGGCTGACGCTGAAGATTTTGCAACTTATTACTGCCAACAGTATAAGAGTTTGTGCTCCTCCTTTTCGGCGGAGGACCA  
 CTTAACTGAGAGTGGTAGTCGCCGACGTCGACCTTTAAACGTTGANTATGACGGTTGTCTCAAAACAGCGAGTGAAAGCCGCTCCCTGGT  
 ThrLeuThrIleSerGlyLeuGlnProGluAspPheAlaThrTyrCysGlnGlnTyrLysSerLeuSerLeuThrPheGlyGlyGlyThrLys  
 72  
 fnu4HI  
 pstI  
 haeIII  
 fnu4HI bbvI mboII  
 101 AGGTGGAGATCAAAACGAACCTGTGGCTGCACCATCTGTCTTTCATCTTCCGCCCATCTGATGAGCAGTTGAAATCTGGAACCTGCCTCTGTGTGCTGCTGCT  
 TCCACCTCTAGTTGCTTGTGACACCGACGTCGAGAGTAGAAGGCGGTAGACTACTCGTCACTTTAGACCTTGACGGGAGACACACACGACCGACGA  
 104 ValGluIleLysArgThrValAlaAlaProSerValPheIlePheProSerAspGluGlnLeuLysSerGlyThrAlaSerValCysLeuLeu  
 137 AsnAsnPheTyrProArgGluAlaLysValGlnTrpLysValAspAsnAlaLeuGlnSerGlyAsnSerGlnGluSerValThrGluGlnAspSerLys  
 xmnI  
 mnlI  
 rsaI  
 mnlI  
 scrFI  
 bstNI  
 201 GAATAACTTCTATCCACAGAGAGGCCAAAGTACAGTGGAAGGTGGATACGCCCTCCCAATCGGGTAACTCCCGAGGAGAGTGTACAGAGCAGGACAGCAAG  
 CTTATTGAAGATAGGCTCTCTCCGGTTTCATGTACCTTCCACCTATTCGGGAGGTAGCCCATTCAGGGTCCCTCTCACAGTGTCTCGTCTGCTGCTC  
 137 AsnAsnPheTyrProArgGluAlaLysValGlnTrpLysValAspAsnAlaLeuGlnSerGlyAsnSerGlnGluSerValThrGluGlnAspSerLys  
 fnu4HI  
 ddeI  
 mnlI bbvI hgaI  
 301 GACAGCACCTACAGCCTCAGCAGCCCTGACCTGAGCTGAGCAAAACAGCAGACTACGACAAACACAAAGTCTACGCCCTGCGAAGTCAACCCATCAGGCGCTGAGCT  
 CTGTCGTGGATGTCGGAGTCGTCGTGGGACTGCGACTCGTTTCCTGATGCTCTTGTGTTTCAGATCGGACGCTTCACTGGGTAGTCCCGGACTCGA  
 170 AspSerThrTyrSerLeuSerSerThrLeuThrLeuSerLysAlaAspTyrGluLysHisLysValTyrAlaCysGluValThrHisGlnGlyLeuSerSer  
 fnu4HI  
 ddeI  
 mnlI bbvI hgaI  
 301 GACAGCACCTACAGCCTCAGCAGCCCTGACCTGAGCTGAGCAAAACAGCAGACTACGACAAACACAAAGTCTACGCCCTGCGAAGTCAACCCATCAGGCGCTGAGCT  
 CTGTCGTGGATGTCGGAGTCGTCGTGGGACTGCGACTCGTTTCCTGATGCTCTTGTGTTTCAGATCGGACGCTTCACTGGGTAGTCCCGGACTCGA  
 170 AspSerThrTyrSerLeuSerSerThrLeuThrLeuSerLysAlaAspTyrGluLysHisLysValTyrAlaCysGluValThrHisGlnGlyLeuSerSer  
 aluI  
 mnlI bsp1286 bspMI mnlI  
 401 CGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTAGAGGGAGAGTGGCCCGACCTGCTCCTCAGT  
 GCGGGCAGTGTTCGGAAGTTGTCCTCTCACAATCTCCCTCTTCACGGGGGTGGACGAGGAGTCA  
 204 ProValThrLysSerPheAsnArgGlyGluCysAsn\*

← V K J K C K →  
 haeIII  
 haeI

FIG. 5

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03414

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : C 12 N 15/00; C 12 P 21/02; A 61 K 37/02; //G 01 N 33/566		
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched <sup>7</sup> Classification System   Classification Symbols IPC <sup>4</sup>   C 12 N; C 12 P; A 61 K Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Cell, vol. 42, no. 1, August 1985, P.J. Maddon et al.: "The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family", pages 93-104, see figure 3, especially fragment T4B (cited in the application) --	1-4,14-16, 19,20,24, 25,50-58,60
X	Proceedings of the National Academy of Sciences of the USA, vol. 84, March 1987 (US) S.J. Clark et al.: "Peptide and nucleotide sequences of rat CD4 (W3/25) antigen: evidence for derivation from a structure with four immunoglobulin-related domains", pages 1649-1653, see figure 3 --	1,2,14,15, 60
X,P	WO, A, 88/01304 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 25 February 1988, see pages 69-75; examples 3-6 --	1-4,14-16, 19,20,24, 25,50-58,60
* Special categories of cited documents: <sup>10</sup> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the International filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "G" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 21st December 1988		Date of Mailing of this International Search Report 13. 01. 89
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer  P.C.G. VAN DER PUTTEN

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,P	Science, vol. 238, 18 December 1987, D.H. Smith et al.: "Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen", pages 1704-1707, see the whole article	1-16,19, 24,25,29, 50-58,60
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V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 59 because they relate to subject matter not required to be searched by this Authority, namely:

please see Rule 39.1 (iv) - PCT:

Methods for treatment of the human or animal body by surgery  
or therapy, as well as diagnostic methods.

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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SA 24961

**For more details about this annex : see Official Journal of the European Patent Office, No. 12/82**